

## Expression of H5 hemagglutinin vaccine antigen in common duckweed (*Lemna minor*) protects against H5N1 high pathogenicity avian influenza virus challenge in immunized chickens



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### ABSTRACT

A synthetic hemagglutinin (HA) gene from the highly pathogenic avian influenza (HPAI) virus A/chicken/Indonesia/7/2003 (H5N1) (Indo/03) was expressed in aquatic plant *Lemna minor* (r*Lemna*-HA). In Experiment 1, efficacy of r*Lemna*-HA was tested on birds immunized with 0.2 µg or 2.3 µg HA and challenged with 10<sup>6</sup> mean chicken embryo infectious doses (EID<sub>50</sub>) of homologous virus strain. Both dosages of r*Lemna*-HA conferred clinical protection and dramatically reduced viral shedding. Almost all the birds immunized with either dosage of r*Lemna*-HA elicited HA antibody titers against Indo/03 antigen, suggesting an association between levels of anti-Indo/03 antibodies and protection. In Experiment 2, efficacy of r*Lemna*-HA was tested on birds immunized with 0.9 µg or 2.2 µg HA and challenged with 10<sup>6</sup> EID<sub>50</sub> of heterologous H5N1 virus strains A/chicken/Vietnam/NCVD-421/2010 (VN/10) or A/chicken/West Java/PWT-WIJ/2006 (PWT/06). Birds challenged with VN/10 exhibited 100% survival regardless of immunization dosage, while birds challenged with PWT/06 had 50% and 30% mortality at 0.9 µg HA and 2.2 µg HA, respectively. For each challenge virus, viral shedding titers from 2.2 µg HA vaccinated birds were significantly lower than those from 0.9 µg HA vaccinated birds, and titers from both immunized groups were in turn significantly lower than those from sham vaccinated birds. Even if immunized birds elicited HA titers against the vaccine antigen Indo/03, only the groups challenged with VN/10 developed humoral immunity against the challenge antigen. None (r*Lemna*-HA 0.9 µg HA) and 40% (r*Lemna*-HA 2.2 µg HA) of the immunized birds challenged with PWT/06 elicited pre-challenge antibody titers, respectively. In conclusion, *Lemna*-expressed HA demonstrated complete protective immunity against homologous challenge and suboptimal protection against heterologous challenge, the latter being similar to results from inactivated whole virus vaccines. Transgenic duckweed-derived HA could be a good alternative for producing high quality antigen for an injectable vaccine against H5N1 HPAI viruses.

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### 1. Introduction

Highly pathogenic influenza A virus subtype H5N1 is an emerging avian influenza (AI) virus that has heightened global concerns as a potential human pandemic threat. H5N1 has not only killed millions of poultry and other birds throughout Asia, Europe, and Africa, but has also resulted in substantial morbidity and mortality in a range of mammalian hosts, including humans [1]. To reduce the economic impact on agriculture and the potential pandemic for humans, development of safe and effective vaccines against H5N1,

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especially vaccines that do not require growth of vaccine antigen in chicken embryos, has been recognized as an essential approach to decrease risk of transmission in poultry and, ultimately, to humans [2,3].

The goal of AI vaccination is the production of an immune response that will protect against the disease and reduce infection and transmission. The ideal AI vaccine should be potent, safe, administered in a single dose, cost effective, and it should enable differentiation of infected from vaccinated animals (DIVA) [3]. Currently, there are two types of licensed poultry AI vaccines: (1) inactivated whole AI virus; and (2) live recombinant virus vectored vaccines with H5 or H7 AI HA gene inserts [4,5]. Conventional inactivated vaccines require growing the AI virus in embryonating chicken eggs (ECE), which necessitates planning production and procurement of ECE plus various levels of containment for production and inactivation of the seed virus [6–9]. Also, replication and hence efficacy of live virus-vectored vaccines can be limited by pre-existing immunity against the vector or insert and thus interfere with production of active immune response [10].

To address the above issues, subunit vaccine development has been explored, including baculovirus [11–14] and, most recently, plant-expression systems [15–18]. In the last two decades, transgenic plants have arisen as safe and cost-effective alternative expression platforms for producing recombinant proteins, with wide applications in animal and human health, diagnostics, and technical enzyme products [19,20]. Among them, common duckweed (*Lemna minor*) is a small, aquatic, higher plant with inherent advantages over other existing microbial, cell culture, and transgenic expression systems, such as desired attributes of rapid clonal growth (36 h doubling time), capability of post-translational modification and secretion of target proteins, high protein yields, and full containment [21]. Numerous proteins have been successfully expressed in *Lemna* including small peptides, Fab fragments, monoclonal antibodies, and large multimeric enzymes [22,23]. Moreover, simple inorganic medium, inexpensive upstream facilities, and scalability make duckweed an attractive production platform for vaccine development [22].

The objective of the present study was to evaluate the efficacy of an H5 HA-derived *Lemna* vaccine in chickens challenged with homologous and heterologous H5N1 HPAI viruses. This recombinant H5 HA demonstrated complete protective immunity against homologous challenge and variable protection against heterologous challenge.

## 2. Materials and methods

### 2.1. Animals

Three-week-of-age (woa) specific-pathogen-free (SPF) White Leghorn chickens from Southeast Poultry Research Laboratory (SEPRL) in-house flocks were used. Each group of birds was housed separately in negative pressured isolators with HEPA-filtered air within the animal biosafety level 2 (ABSL-2) facilities of SEPRL during vaccination period, and they were subsequently transferred to animal biosafety level 3 enhanced (ABSL-3E) facilities, housed in negative pressure HEPA-filtered isolators for challenge period. Feed and water were provided *ad libitum* throughout the experiment. All procedures were performed according to the requirements of the protocol approved by the Institutional Laboratory Animal Care and Use Committee.

### 2.2. Lemna-derived HA vaccine

An adjuvanted non-purified full length HA subunit protein derived from clade 2.1.1 A/chicken/Indonesia/7/2003 (H5N1)

(Indo/03) HPAI virus was used. The proteolytic cleavage site of the expressed H5 HA was changed from a high to low pathogenicity motif to allow exclusion from USA Select Agent rules. The HA sub-unit was produced in *L. minor* using *Lemna* Expression System (LEX System<sup>TM</sup>, Biolex Therapeutics, Pittsboro, NC). Crude tissue extraction from *Lemna* transgenic line was performed at 4 °C. Briefly, 100 g of frozen biomass was mixed with 200 ml extraction buffer (50 mM NaPO<sub>4</sub>, 0.3 M NaCl, 10 mM EDTA, pH 7.4, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)) and homogenized in Waring blender (Fisher Scientific International Inc., Hampton, NH) with 20 s burst for 4 times and 10–20 s cooling in between. The homogenate was centrifuged at 14,000 × g for 30 min at 4 °C and clarified by passing through cheese cloth to remove any large debris and subsequently through cellulose acetate filter (0.22 µm). Total soluble protein was determined using the Bradford assay with bovine serum albumin as a standard.

Crude tissue extract from *Lemna* transgenic line was formulated in water-in-oil emulsion (r*Lemna*-HA) and used as a vaccine. Briefly, one part aqueous antigen was emulsified in four parts oil phase, which contained 36 ml Drakeol 6 VR pharmaceutical grade mineral oil (Penreco, Karns City, PA), 3 ml Arlacel 80 (7.5% sorbitan mono-oleate, Sigma-Aldrich), and 1 ml Tween 80 (2.5% polysorbate, Sigma-Aldrich). Vaccines were homogenized using a Waring blender with the following mixing speeds: 1 min on low speed, 1 min rest, 1 min on low speed, 1 min rest, and 30 s on high speed [24]. Vaccines were incubated for 1 h at 37 °C and stored at 4 °C until use.

Western blot using monoclonal anti-H5 HA of A/Vietnam/1203/04 (VN/04) influenza virus confirmed expression of HA with the expected molecular weight at 77 kDa (data not shown). Hemagglutination assay was performed to evaluate functionality of *Lemna*-derived HA, showing a hemagglutination activity of 400,000 HA units (HAU)/ml, which was considerably higher than both purified recombinant HA control at 87 µg/ml and inactivated H5N1 AI strain Indo/03 at 10<sup>8.5</sup> mean chicken embryo infectious doses (EID<sub>50</sub>). Immunofluorescence assay using monoclonal anti-H5 HA of VN/04 confirmed expression of HA in apoplast of the plant tissue, which was the initial target cellular location for HA expression (data not shown). The expressed HA of Indo/03 was estimated to be 12% of total soluble protein based on densitometry analysis of SDS-PAGE, and the overall yield of HA in *Lemna* plant was calculated to be ~280 mg/kg of frozen biomass with specific HA titer at 2.875 HAU/µg HA.

### 2.3. Viruses

In Experiment 1, the same seed virus (clade 2.1.1 Indo/03) was used for challenge. In Experiment 2, clade 2.3.2.1 A/chicken/Vietnam/NCVD-421/2010 (H5N1) (VN/10) and clade 2.1.3.2 A/chicken/West Java/PWT-WIJ/2006 (H5N1) (PWT/06) viruses were used for challenge. The latter virus was an antigenic variant that was poorly protected by inactivated H5N1 HPAI (clade 2.1) vaccines and other H5 vaccines, and recombinant fowl poxvirus with HA gene insert from A/turkey/Ireland/1983 (H5N8) [25]. Viruses were propagated and titrated by allantoic sac inoculation of 9-day-of-age ECE by standard methods [26].

### 2.4. Viral RNA quantification in oropharyngeal swabs

Oropharyngeal swabs were processed by quantitative real-time RT-PCR (qRT-PCR) to determine viral RNA titers. Cycle threshold (Ct) values were converted to equivalent infectious titers in ECE based on regression line produced using a challenge virus dilution series [27]. Briefly, RNA was extracted from oropharyngeal swab material by adding 250 µl of swab medium to 750 µl of Trizol<sup>®</sup> LS (Invitrogen Inc., Carlsbad, CA), followed by

vortexing, incubation at room temperature for 10 min, and then 200 µl of chloroform was added. The samples were vortexed again, incubated at room temperature for 10 min, and then centrifuged for 15 min at approximately 12,000 × g. The aqueous phase was collected and RNA was extracted with MagMAX™-96 Al/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX) following the manufacturer's instructions using the KingFisher™ magnetic particle processing system (Thermo Scientific, Waltham, MA). The challenge virus was used to produce the RNA for the quantitative standard. Allantoic fluid virus stocks were diluted in brain heart infusion (BHI) medium and titrated in ECE at the time of dilution as per standard methods [28]. Whole virus RNA was extracted from ten-fold dilutions of titrated virus as described for swab material. qRT-PCR for the influenza matrix gene was performed as previously described [27]. Virus titers in samples were calculated based on the standard curves, either calculated by the SmartCycler® II (Cepheid, Inc., Sunnyvale, CA) software or extrapolation of the standard curve equation.

## 2.5. Serology

Hemagglutinin inhibition (HI) assays were performed to evaluate antibody levels in vaccinated birds both pre- and post-challenge. The vaccine seed virus (Indo/03) and the challenge viruses (VN/10 and PWT/06) were tested as antigens. The antigens were prepared as previously described [29], and the HI assays were performed according to standard procedures [30]. Titers were expressed as log<sub>2</sub> geometric mean titers (GMT-log<sub>2</sub>).

## 2.6. Statistical analysis

Mortality and number of birds shedding virus were tested for statistical significance with Fisher's exact test. Significant difference for mean viral titers between groups was analyzed using Mann-Whitney test. Antibody levels were tested for statistical significance with Kruskal-Wallis and Mann-Whitney tests (GraphPad Prism™ Version 5 software). A P-value of <0.05 was considered to be significant.

## 2.7. HA sequence analysis

HA amino acid sequences of the isolates used in this study were obtained from GenBank and Influenza Research Database (<http://www.fludb.org/brc/home.do?decorator=influenza>). Comparison of

HA amino acid sequences was performed using Clustal W method with MegAlign (DNA STAR Lasergene 8, Madison, WI).

## 2.8. Experimental design

### 2.8.1. Experiment 1. Efficacy against homologous H5N1 HPAI virus challenge

Thirty chickens were randomly distributed into three vaccine groups (*n* = 10/group) (Table 1). At 3 weeks of age (woa), chickens were vaccinated with rLemna-HA at two dosage levels chosen based on a previous study of baculovirus-expressed HA [12]: 655 HAU or 0.2 µg HA (group 2) and 6550 HAU or 2.3 µg HA (group 3). Negative control birds (group 1) were inoculated with adjuvanted Lemna wild type protein without HA insert (sham). Vaccines were intramuscularly administered in the leg (0.3 ml/dose).

At 3 weeks post-vaccination (6 woa), chickens were bled for serum collection and were challenged intranasally with H5N1 HPAI virus Indo/03 at 10<sup>6</sup> EID<sub>50</sub>/0.1 ml per bird. The inoculum titer was subsequently verified as 10<sup>6.3</sup> EID<sub>50</sub>/0.1 ml by back titration in ECE. Chickens were monitored daily for clinical signs and mortality. Oropharyngeal swabs were collected at 2 and 4 days post-challenge (dpc) in BHI medium with antibiotics (100 µg/ml gentamicin, 100 units/ml penicillin, and 5 µg/ml amphotericin B). At the end of the experiment (14 dpc, 8 woa), surviving birds were bled for serum collection and humanely euthanized by intravenous sodium pentobarbital (100 mg/kg body weight).

### 2.8.2. Experiment 2. Efficacy against heterologous H5N1 HPAI virus challenge

Sixty chickens were randomly distributed into six vaccine groups (*n* = 10/group) (Table 2). For each challenge virus, 3-woa chickens were vaccinated with rLemna-HA at two dosage levels: 2500 HAU or 0.9 µg HA (groups 2 and 5) and 6250 HAU or 2.2 µg HA (groups 3 and 6). Negative control birds (groups 1 and 4) were inoculated with adjuvanted Lemna wild type protein without HA insert (sham). Vaccines were intramuscularly administered in the leg (0.3 ml/dose).

At 3 weeks post-vaccination (6 woa), chickens were bled for serum collection and were challenged intranasally with either H5N1 VN/10 (groups 1, 2 and 3) or PWT/06 (groups 4, 5 and 6) at 10<sup>6</sup> EID<sub>50</sub>/0.1 ml per bird. The inoculum titers were further verified for both viruses as 10<sup>6.1</sup> EID<sub>50</sub>/0.1 ml by back titration in ECE. Sampling was performed as for Experiment 1, with the only difference that oropharyngeal swabs were only collected on 2 dpc.

**Table 1**

Clinical and serological data from 3-woa White Leghorn chickens vaccinated with the adjuvanted crude *Lemna* extract and challenged 3 weeks later with 10<sup>6</sup> EID<sub>50</sub> A/chicken/Indonesia/7/2003 (H5N1) HPAI virus.

Group	Vaccines	Mortality (MDT <sup>a</sup> )	Viral RNA detection on oropharyngeal swabs <sup>†</sup> (log <sub>10</sub> EID <sub>50</sub> titer/ml)		HI serology (GMT-log <sub>2</sub> ) <sup>‡</sup> against A/chicken/Indonesia/7/2003	
			2 dpc	4 dpc	Pre-challenge	Post-challenge
1	<i>Lemna</i> wild type (sham)	10/10 <sup>A</sup> (2.1)	10/10 <sup>A</sup> (6.9 <sup>a</sup> )	nd	0/10	nd
2	r <i>Lemna</i> -HA (0.2 µg HA)	0/10 <sup>B</sup>	4/10 <sup>B</sup> (3.1 <sup>b</sup> )	0/10 (2.8)	9/10 (4.5 ± 1.3) <sup>a</sup>	10/10 (7.1 ± 1.4) <sup>b</sup>
3	r <i>Lemna</i> -HA (2.3 µg HA)	0/10 <sup>B</sup>	1/10 <sup>B</sup> (2.9 <sup>b</sup> )	0/10 (2.8)	10/10 (6.9 ± 1.0) <sup>c</sup>	10/10 (8.2 ± 0.8) <sup>b</sup>

MDT, mean death time; dpc, days post-challenge; HI, hemagglutination inhibition; GMT, geometrical mean titer.

<sup>a</sup> #dead birds × dpc/total dead birds (expressed as dpc).

<sup>†</sup> #positive/total. Viral shedding determined by qRT-PCR. qRT-PCR samples with Ct values >38 were confirmed by PCR for the NS1 gene. Titer values for the NS1-positive samples were calculated from the Smart Cycler equation. RT-PCR detection limit was 2.9 ng EID<sub>50</sub>/ml. For statistical purposes, negative samples were given a value of 2.8 log EID<sub>50</sub>/ml.

<sup>‡</sup> #positive/total.

<sup>A</sup> GMT (log<sub>2</sub>) ± standard deviation. It includes only positive birds.

nd, no data is available because no birds survived.

Different superscript uppercase letters denote significant difference for mortality and for number of positive birds on qRT-PCR among groups; Fisher's exact test, *P* < 0.05.

Different superscript lowercase letters inside the parenthesis denote significant difference for mean viral titers among groups; Mann-Whitney test, *P* < 0.05.

Different superscript lowercase letters outside the parenthesis denote significant difference for HI titers among groups. Kruskal-Wallis test, *P* < 0.05; Mann-Whitney test, *P* < 0.05.

### 3. Results

#### 3.1. Experiment 1. Efficacy against homologous H5N1 HPAI virus challenge

All birds sham vaccinated with *Lemna* wild type died within 2 days of challenge with Indo/03 (Table 1). By contrast, the vaccinated groups exhibited 100% survival at both 0.2 and 2.3 µg HA doses.

Viral RNA titers were determined on oropharyngeal swabs of 2 and 4 dpc by qRT-PCR (Table 1). All sham chickens, i.e. vaccinated with *Lemna* wild type, were positive on 2 dpc, with an overall mean titer of  $10^{6.9}$  EID<sub>50</sub>/ml. Among the r*Lemna*-HA immunized groups, viral shedding was reduced dramatically to just above detection limit ( $10^{2.9}$  EID<sub>50</sub>/ml) on 2 dpc, with 4/10 positive birds in 0.2 µg HA and only 1/10 positive birds in 2.3 µg HA groups. By 4 dpc, virus was completely cleared in both HA immunized groups. Both vaccinated groups had significant fewer birds shedding virus and significant lower titers than the sham vaccinated group.

Pre-challenge (0 dpc) and termination (14 dpc) sera were processed for HI assay to evaluate antibody levels against Indo/03 vaccine seed and challenge virus (Table 1). None of the sham vaccinated birds developed humoral immunity to Indo/03 strain by the day of challenge. In contrast, 90–100% of the birds immunized with either dosage of r*Lemna*-HA elicited HI titers to Indo/03 antigen, both pre- and post-challenge. In particular, mean HI titers against Indo/03 significantly increased from  $\log_2 4.5 \pm 1.3$  to  $7.1 \pm 1.4$  GMT in 0.2 µg HA immunized birds, and from  $\log_2 6.9 \pm 1.0$  to  $8.2 \pm 0.8$  GMT in 2.3 µg HA immunized birds. Pre-challenge titers of 2.3 µg HA were significantly higher than pre-challenge titers of 0.2 µg HA, although post-challenge results were not significantly different between them.

#### 3.2. Experiment 2. Efficacy against heterologous H5N1 HPAI virus challenge

All chickens in sham groups challenged with VN/10 (group 1) or PWT/06 (group 4) died within 2 days (Table 2). Birds immunized at either 0.9 or 2.2 µg HA of r*Lemna*-HA and challenged with VN/10 exhibited 100% survival. On the contrary, birds in the r*Lemna*-HA group challenged with PWT/06 had 50% and 70% survival at 0.9 and 2.2 µg HA, respectively, being significantly different from survival of the sham group.

Viral RNA titers were determined on oropharyngeal swabs of 2 dpc by qRT-PCR (Table 2). All sham vaccinated chickens were positive, with an overall mean titer of  $10^{8.0}$  EID<sub>50</sub>/ml and  $10^{7.5}$  EID<sub>50</sub>/ml after challenge with VN/10 and PWT/06, respectively. Regardless of the challenge virus, viral shedding was not totally prevented among

the immunized groups as 100% and 90% of the birds were shedding virus on 2 dpc from the 0.9 and 2.2 µg HA vaccine groups, respectively. When comparing mean viral titers among groups challenged with VN/10, titers from sham vaccinated birds were significantly higher than those from either of the immunized groups, although titers from 0.9 and 2.2 µg HA immunized birds were statistically similar. In the case of PWT/06, titers from sham vaccinated birds were significantly higher than those from either of the immunized groups, and those from 0.9 µg HA vaccinated birds were in turn significantly higher than those from 2.2 µg HA vaccinated birds; i.e. suggested vaccine dose dependent reduction in challenge virus replication and shedding from oropharynx.

Pre-challenge (0 dpc) and termination (14 dpc) sera were processed for HI assays to evaluate antibody levels against the vaccine seed virus (Indo/03) and the challenge viruses (VN/10 or PWT/06) (Table 3, Fig. 1). Ninety and 100% of the immunized birds with either dosage of r*Lemna*-HA elicited HI titers to the vaccine antigen Indo/03, both pre- and post-challenge. Interestingly, birds immunized with r*Lemna*-HA at 2.2 µg HA did not have a significant difference in antibody titers as compared to their corresponding 0.9 µg HA groups. Almost all (70% and 90%) immunized birds challenged with VN/10 developed humoral immunity against the challenge antigen, and the pre-challenge HI titers of 0.9 µg HA group ( $\log_2 3.5 \pm 0.7$  GMT) were statistically lower than those of 2.2 µg HA group ( $\log_2 4.4 \pm 1.7$  GMT). On the contrary, none of the birds challenged with PWT/06 and immunized with r*Lemna*-HA at 0.9 µg HA elicited pre-challenge antibody titers against the challenge virus, although 5 out of 10 birds survived; and only 40% of the birds immunized with r*Lemna*-HA at 2.2 µg HA elicited very low pre-challenge antibody titers ( $\log_2 3.5$  GMT). For 2.2 µg HA group, three of these four birds with pre-challenge titers survived, while the fourth one did not survive but only had a minimal detected pre-challenge titer of  $\log_2 3$  GMT. In addition, four birds with no pre-challenge titers were among the seven survivors. Almost all the surviving birds had developed an anamnestic response by the end of the experiment.

#### 3.3. Amino acid comparison of HA sequences

Comparison of HA amino acid sequences was performed to account for differences observed in protection conferred by r*Lemna*-HA vaccine (Indo/03) when using the challenge viruses VN/10 and PWT/06. The HA amino acid similarities between the Indo/03 vaccine and the challenge viruses were 95.0% with PWT/06 and 93.5% with VN/10 (93.5%) (Table 4). However, the HA1 sequences were very similar: 91.7% between PWT/06 and Indo/03, and 92% between VN/10 and Indo/03 (Table 4).

**Table 2**

Clinical and serological data from 3-week-old White Leghorn chickens vaccinated with the adjuvanted crude *Lemna* extract and challenged 3 weeks later with  $10^6$  EID<sub>50</sub> of either A/chicken/Vietnam/NCVD-421/2010 or A/chicken/West Java/PWT-WIJ/2006 H5N1 HPAI viruses.

Group	Vaccines	Challenge virus	Mortality (MDT <sup>*</sup> )	Viral RNA detection on 2 dpc oropharyngeal swabs <sup>†</sup> ( $\log_{10}$ EID <sub>50</sub> titer/ml)
1	<i>Lemna</i> wild type (sham)	VN/10	10/10 (2.0)	$10/10^A$ (8.0 <sup>a</sup> )
2	r <i>Lemna</i> -HA (0.9 µg HA)	VN/10	0/10	$10/10^A$ (5.1 <sup>b</sup> )
3	r <i>Lemna</i> -HA (2.2 µg HA)	VN/10	0/10	$9/10^A$ (4.6 <sup>b</sup> )
4	<i>Lemna</i> wild type (sham)	PWT/06	10/10 <sup>A</sup> (2.0)	$10/10^A$ (7.5 <sup>a</sup> )
5	r <i>Lemna</i> -HA (0.9 µg HA)	PWT/06	5/10 <sup>B</sup> (2.6)	$10/10^A$ (5.0 <sup>b</sup> )
6	r <i>Lemna</i> -HA (2.2 µg HA)	PWT/06	3/10 <sup>B</sup> (7.0)	$9/10^A$ (3.7 <sup>c</sup> )

VN/10, A/chicken/Vietnam/NCVD-421/2010; PWT/06, A/chicken/West Java/PWT-WIJ/2006; MDT, mean death time; dpc, days post-challenge.

\* #dead birds × dpc/total dead birds (expressed as dpc).

† #positive/total. Viral shedding determined by qRT-PCR. qRT-PCR samples with Ct values >38 were confirmed by PCR for the NS1 gene. Titer values for the NS1-positive samples were calculated from the Smart Cycler equation. Negative threshold values ( $\log_{10}$  EID<sub>50</sub>/ml) =  $\leq 3.6$  for A/chicken/Vietnam/NCVD-421/2010 (H5N1) and  $\leq 3.1$  for A/chicken/West Java/PWT-WIJ/2006 (H5N1). For statistical purposes, negative samples were given a value of 0.1 log EID<sub>50</sub>/ml below the detection limit.

Different superscript uppercase letters denote significant difference for mortality and for number of positive birds on qRT-PCR among groups with the same challenge virus; Fisher's exact test,  $P < 0.05$ .

Different superscript lowercase letters denote significant difference for mean viral titers among groups with the same challenge virus; Mann-Whitney test,  $P < 0.05$ .

**Table 3**

Serological data from 3-wk old White Leghorn chickens vaccinated with the adjuvanted crude *Lemna* extract and challenged 3 weeks later with  $10^6$  EID<sub>50</sub> of either A/chicken/Vietnam/NCVD-421/2010 or A/chicken/West Java/PWT-WIJ/2006 H5N1 HPAI viruses.

Group	Vaccines	Challenge virus	HI serology* (GMT-log <sub>2</sub> )†					
			A/ck/Indonesia/7/2003		A/ck/Vietnam/NCVD-421/2010		A/ck/West Java/PWT-WIJ/2006	
			Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
1	<i>Lemna</i> wild type (sham)	VN/10	–	–	0/10 <sup>A</sup> (<3) <sup>a</sup>	nd	–	–
2	r <i>Lemna</i> -HA (0.9 µg HA)	VN/10	10/10 <sup>A</sup> (5.6 ± 1.3) <sup>b</sup>	10/10 <sup>A</sup> (8.1 ± 1.0) <sup>a</sup>	7/10 <sup>B</sup> (3.5 ± 0.7) <sup>ab</sup>	10/10 <sup>A</sup> (7.0 ± 0.8) <sup>a</sup>	–	–
3	r <i>Lemna</i> -HA (2.2 µg HA)	VN/10	10/10 <sup>A</sup> (6.9 ± 1.4) <sup>b</sup>	10/10 <sup>A</sup> (8.8 ± 1.8) <sup>a</sup>	9/10 <sup>B</sup> (4.4 ± 1.7) <sup>b</sup>	10/10 <sup>A</sup> (7.3 ± 1.6) <sup>a</sup>	–	–
4	<i>Lemna</i> wild type (sham)	PWT/06	–	–	–	–	0/10 <sup>A</sup> (<3) <sup>a</sup>	nd
5	r <i>Lemna</i> -HA (0.9 µg HA)	PWT/06	9/10 <sup>A</sup> (6.1 ± 1.4) <sup>a</sup>	5/5 <sup>A</sup> (9.0 ± 1.6) <sup>a</sup>	–	–	0/10 <sup>A</sup> (<3) <sup>a</sup>	5/5 <sup>A</sup> (6.2 ± 1.3) <sup>a</sup>
6	r <i>Lemna</i> -HA (2.2 µg HA)	PWT/06	10/10 <sup>A</sup> (6.6 ± 1.3) <sup>a</sup>	7/7 <sup>A</sup> (9.1 ± 2.2) <sup>a</sup>	–	–	4/10 <sup>A</sup> (3.5 ± 0.6) <sup>a</sup>	6/7 <sup>A</sup> (5.7 ± 2.0) <sup>a</sup>

VN/10, A/chicken/Vietnam/NCVD-421/2010; PWT/06, A/chicken/West Java/PWT-WIJ/2006; HI, hemagglutination inhibition; GMT, geometrical mean titer.

\* #positive/total.

† GMT (log<sub>2</sub>) ± standard deviation. It includes only positive birds.

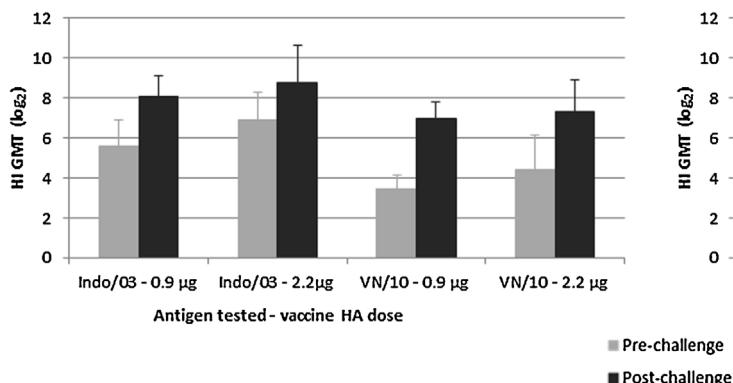
nd, no data is available because all birds died before 14 dpc.

– not performed.

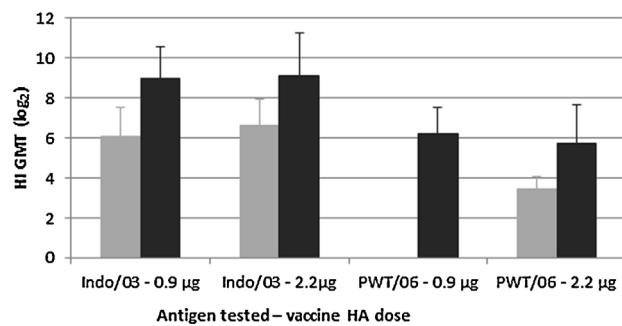
Different superscript uppercase letters denote significant difference for number of positive birds on HI among groups with the same challenge virus; Fisher's exact test,  $P < 0.05$ .

Different superscript lowercase letters denote significant difference for HI titers among groups with the same challenge virus. Kruskal-Wallis test,  $P < 0.05$ ; Mann-Whitney test,  $P < 0.05$ .

### A VN/10 challenge



### B PWT/06 challenge



**Fig. 1.** Serological responses pre- (0 dpc) and post-challenge (14 dpc) of White Leghorn chickens vaccinated with the adjuvanted crude *Lemna* extract and challenged 3 weeks later with  $10^6$  EID<sub>50</sub> of either (A) A/chicken/Vietnam/NCVD-421/2010 (VN/10) or (B) A/chicken/West Java/PWT-WIJ/2006 (PWT/06) H5N1 HPAI viruses. Results are expressed as GMT (log<sub>2</sub>) ± SD.

Interestingly, although both challenge viruses had approximately the same HA1 similarity to the vaccine, they were 12% divergent to each other (data not shown). The amino acid sequence of the HA1 subunit showed a total of 30 substitutions in PWT/06 and 28 substitutions in VN/10 when compared to Indo/03 sequence (Supplemental Fig. 1), which corroborates the HA1 similarity among the sequences (Table 4). However, 10 of the 28 substitutions in PWT/06

were located in the above mentioned antigenic sites, while this was the case of only 5 of the 30 substitutions in VN/10 (Supplemental Fig. 1). In particular, N125K and Q131R were in PWT/06 but not in VN/10. Also, P197S, D199N, A200V, and A201E were observed in PWT/06, while A200E was the only substitution present in the 220-loop region of VN/10.

**Table 4**

HA and HA1 sequence similarities between the vaccine seed virus A/chicken/Indonesia/7/2003 and challenge viruses A/chicken/Vietnam/NCVD-421/2010 and A/chicken/West Java/PWT-WIJ/2006.

Viruses	Amino acid similarity with Indo/03 (%)	
	HA	HA1
VN/10	93.5	92.0
PWT/06	95.0	91.7

HA, hemagglutinin; HA1, hemagglutinin subunit 1; Indo/03, A/chicken/Indonesia/7/2003; VN/10, A/chicken/Vietnam/NCVD-421/2010; PWT/06, A/chicken/West Java/PWT-WIJ/2006.

## 4. Discussion

During the last decade, subunit vaccines have been explored as a suitable immunization strategy in AI control to overcome the limitations of the currently licensed vaccines (*i.e.*, inactivated and live recombinant vaccines) by producing the vaccine antigen in a system other than ECE and the vaccine being amenable to identification of infected animals in the vaccinated population (*i.e.*, DIVA). The objective of this study was to evaluate efficacy of r*Lemna*-HA vaccine which is proposed as a safe, effective, cost-efficient and scalable production platform to create plant-derived HA vaccines.

Protective efficacy of rLemna-HA was evaluated in immunized 3-woa chickens challenged with homologous Indo/03 H5N1 HPAI virus (Experiment 1) or heterologous VN/10 and PWT/06 H5N1 HPAI viruses (Experiment 2). A single immunization of rLemna-HA conferred complete clinical protection against homologous H5N1 HPAI strain even at the lowest dose (0.2 µg HA), and against heterologous VN/10 HPAI virus at both doses (0.9 µg HA and 2.2 µg HA). However, only partial clinical protection was achieved against challenge with antigenic variant PWT/06 strain, which was also shown to be poorly protected by inactivated vaccines derived from A/Turkey/Wisconsin/1968 (H5N9), A/Turkey/England/N28/1973 (H5N2), A/Chicken/Mexico/232/1994 (H5N2), A/chicken/Legok/2003 (H5N1), A/Goose/Guangdong/1/1996 (H5N1), and A/duck/Vietnam/2004 (H5N3) [25]. By contrast, the 100% protection observed by rLemna-HA vaccine against Indo/03 and VN/10 H5N1 HPAI viruses were equivalent or superior to previous reports on protection provided by HA expressed in baculovirus- and other plant-based expression systems when challenged with HPAI viruses. Consistent serum antibody response and complete protection in SPF chickens against challenge with lethal H5 and H7 strains were achieved with baculovirus-derived 250 HAU–1.0 µg HA [12]. Using higher HA doses than in our study, immunization of ferrets with *Nicotiana benthamiana* plant-derived HA at 45- and 90-µg HA doses elicited antibodies and conferred protection against challenge with a homologous H5N1 HPAI virus [16].

A dramatic reduction in number of birds shedding and the titer of oral shedding with homologous challenge virus Indo/03 was observed at 2 dpc, and the virus was completely cleared by 4 dpc. In contrast, 90% (2.2 µg HA) to 100% (0.9 µg HA) of the immunized birds challenged with VN/10 or PWT/06 were shedding challenge virus at 2 dpc, although significant reduction in the titers of virus shed were noted in almost all the vaccines compared to sham immunized birds. In the field, since infection with heterologous HPAI virus would be asymptomatic, the virus could easily go unnoticed while being spread from farm to farm even when applying vaccination. Whether higher HA doses of rLemna-HA vaccine would provide better protection from mortality and effectively prevent viral shedding warrants further study. Previously, vaccination with baculovirus-derived 1.0 µg HA reduced cloacal shedding below detectable levels in SPF chickens challenged with lethal dose of H5N2 HPAI virus, although optimal reduction in oral and cloacal shedding was only achieved with 5.0 µg HA immunization dose [12]. Similarly, the average virus titer in nasal washes was significantly lower compared to the control group in ferrets immunized with plant-derived HA with doses as high as 90 and 45 µg HA [16].

Serological data supported our findings in clinical protection and prevention of virus shedding. In Experiment 1 with homologous challenge virus, birds immunized with Indo/03 and subsequently challenged with the seed strain developed serum HI antibody titers that were protective against clinical signs and death. In Experiment 2 with heterologous challenge virus, although all the vaccinated birds elicited HI antibodies against the vaccine strain, only the groups that developed humoral immunity against the corresponding challenge virus survived, and on an individual basis, 19 of 20 chickens with HI antibodies against the heterologous challenge virus survived virulent challenge. Such group- and individual-level data supports a positive predictor value for survival if individual chickens had serum HI antibody titers against the challenge virus, as has been previously demonstrated [25]. However, at the individual level there were 13 individuals that lacked HI titers, but which survived heterologous challenge. This scenario calls for further understanding of the immune responses to vaccines for longer periods of time, and for the need of using HI antibody titers together with other relevant immunological parameters like mucosal and cell-mediated immunity to predict protection.

Previous reports indicate that HA similarity between vaccine and challenge virus is often associated with higher vaccine efficacy [5,25,31–33]. In the current study, the HA amino acid similarity between PWT/06 and the Indo/03 vaccine (95.0%) was unexpectedly higher than between VN/10 and Indo/03 (93.5%) (Table 4). Interestingly, although both challenge viruses had about the same HA1 similarity to the vaccine, they were 12% divergent to each other. Such divergence was accounted by the substitutions observed in the antigenicity-associated sites formed by the 190-helix, 220-loop, and 130-loop proximal to the HA receptor-binding domain which have been recognized as determinants of receptor binding preference [34–37]. In this way, substitutions in the above mentioned regions were more numerous and critical in PWT/06 compared to VN/10, accounting for the greater antigenic drift of the HA for PWT/06 than VN/10, from the Indo/03 vaccine insert. These findings corroborate not only the HA1 similarity but also the different protection conferred by the vaccine when using these different challenge viruses. The HA1 subunit is the globular tip of the HA that contains the receptor for binding to the host cell surface [38], and thus, host antibodies are directed to this globular tip in order to prevent binding and entry of the virus [39]. Mutational changes in this segment often affect the antigenicity of the virus and reaction with host antibodies, giving the virus an “escape” mechanism from antibodies that allow efficient binding and entry to the cell [39]. Although there is an association between genetic and antigenic evolution, punctual genetic differences, especially immediately adjacent to the receptor binding site, can lead to a substantial antigenic change or drift [40,41], as recently observed for Indonesian H5N1 antigenic variants [41] as well as pandemic H1N1 avian isolates [42]. Overall, these studies underline the relevance of genetic similarity between the vaccine and the virus challenge on protective efficacy, but they emphasize that other factors such as the antigenic relatedness, virulence of the challenge virus, antigen doses, or use of different adjuvant systems may also influence substantially vaccine effectiveness [43]. Therefore, clinical protection and prevention of virus shedding by the rLemna-HA vaccine against lethal H5N1 HPAI disease does not necessarily require stringent identity between the HA of the vaccine and challenge strains, but sharing significant sequence identity with the challenge virus would be optimal, as previously stated [12]. Low similarity can be overcome by higher antigen doses and/or multiple vaccinations, and therefore be protective to challenge against heterologous viruses [3,44,45].

The present study demonstrates that transgenic duckweed-derived HA can produce high quality antigen for an injectable vaccine against H5N1 HPAI viruses. Such recombinant H5 HA conferred complete protective immunity against homologous challenge, although it provided variable and with one challenge virus suboptimal protection against heterologous challenge which was likely associated with divergent antigenic relatedness between vaccine and challenge viruses. Further studies are needed to address whether higher HA doses or multiple vaccinations would confer protective immunity against heterologous challenge viruses and prevent shedding.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2015.05.076>

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